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Mini-review

# Biomarker discovery in asthma and COPD: Application of proteomics techniques in human and mice

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ABSTRACT

The use of advanced proteomics approaches in the search for biomarkers in chronic lung diseases, such as asthma and COPD, is rather limited. Asthma and COPD are complex disorders, which can be subdivided into several phenotypes. This results in a heterogeneity of differential expressed biological molecules. Furthermore, genetic differences between animals and humans make 'translation' of possible biomarkers challenging. Yet, the improved sensitivity and high throughput of proteomic techniques could be an important asset for (new) protein biomarker discovery in either human or animal models. We have reviewed the literature that reported the use of different proteomics approaches performed on samples obtained from humans and murine models in asthma and COPD research for the discovery of new biomarkers of diseases, biomarkers of sensitization or for the refinement of treatment. There is an increasing trend in the use of proteomics to explore new biomarkers of asthma or COPD. Although several murine models have been developed to study these lung diseases, and proteomics studies have been performed, 'translation' of identified candidate biomarkers into clinical studies is often lacking.



## Contents



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## 1. Introduction

A rapid increase in the use of proteomics in different research domains such as cancer, invertebrate immunology and plant physiology was made possible thanks to the completion of several genomes, technical advances in instrumentation (increased sensitivity) and methods (purification, separation, identification) and the development of new bioinformatical tools [1–5]. In 2013, almost 5000 papers were published using a proteomics technique (*Pubmed search: Publication date: 2013; Keyword: Proteomics*). Yet, the use of advanced proteomics approaches in the search for biomarkers in chronic lung diseases, such as asthma and COPD, is rather limited. This limitation is attributable to multiple factors. First, asthma and COPD are complex disorders which are subdivided into several phenotypes [6–8]. This heterogeneity results in differential expression of biological effector molecules, namely proteins and peptides. Moreover, human samples are inherently characterized by a larger heterogeneity as compared to murine samples due to a different genetic background. Finally, ethically and practically, it is not feasible to obtain large numbers of human samples hampering large proteomic studies [9].

Nevertheless, the sensitivity and high throughput of proteomics techniques could be useful to discover (new) biomarker proteins in humans or animal models, previously not associated with a disease phenotype, at a time point much earlier than current existing diagnostic tools can do [10]. Despite efforts to develop animal models to model different human diseases, translation of (proteomics) research evidence from animals to humans is difficult and time consuming. Only one out of three animal studies evaluating medical interventions for human diseases have been replicated in clinical trials with an average time span of seven years to complete the translation from animals to humans [11].

We have reviewed the literature that reported the use of different proteomics approaches performed on samples obtained from humans and murine models in asthma and COPD research for the discovery of new biomarkers of diseases, biomarkers of sensitization or for the refinement of treatment.

Asthma affects more than 300 million people worldwide, whereas COPD is predicted to be the third most leading cause of death by 2030 (WHO; www.who.int).

Asthma, characterized by a reversible airway obstruction, a non-specific airway hyperreactivity and an airway inflammation, is orchestrated by the interplay of numerous cells, from both the innate immune system (e.g. dendritic cells, macrophages, mast cells, eosinophils and neutrophils) and the adaptive immune system (T cells and B cells) [12–14]. Clinical manifestations of asthma are episodic wheezing, coughing, shortness of breath and non-specific hyperreactivity to e.g. cold air or cigarette smoke. Asthma can be subdivided in different classes (allergic asthma, non-allergic asthma, occupational asthma, etc.). Although different phenotypes of asthma have already been distinguished, the exact molecular mechanisms underlying the disease remain unclear [14].

Chronic obstructive pulmonary disease (COPD) is characterized by a progressive airflow limitation that is partially reversible, a chronic lung inflammation and systemic effects [6]. Although the main risk factor to develop COPD is smoking tobacco, with more than 90% of COPD patients having a smoking history, other factors such as maternal smoking, childhood asthma and outdoor air pollution have recently been associated with the development of COPD [15].

The prevalence of both asthma and COPD is increased during the last decades, indicating that current state-of-the art techniques for diagnosis and management are suboptimal [16]. Current diagnostic tools such as spirometry fail to pinpoint the early onset of chronic lung diseases resulting in late diagnosis when adverse clinical symptoms have already been established. Moreover, the few existing molecular markers lack in sensitivity and specificity (e.g. nitric oxide, eosinophil cationic protein and eosinophil peroxidase) [16,17].

## 2. Proteomics approaches in asthma and COPD

Clinically, it has been estimated that almost 10% of the genome is directly involved in disease pathogenesis. Therefore, detecting distinct protein biomarkers, encoded by the genome, in certain pathologies can contribute to disease detection, monitoring disease progression and response to treatment [18].

So far, most proteomic studies performed in either patient cohorts or murine models of asthma and COPD have investigated established disease (Tables 1 and 2). Several of the differentially expressed proteins in these studies, could be linked with hallmarks of allergic asthma such as airway hyperreactivity, lung inflammation [19–21] and remodeling [22–24]. Proteomic changes due to long term cigarette smoke exposure in murine models could be related to lung damage and smoke-induced lung irritation [25,26]. Proteomic screening in humans suffering from COPD most often focused on samples obtained from the respiratory system, such as bronchoalveolar lavage (BAL), lung tissue or cells, epithelial lining fluid or induced sputum. Some studies have also analyzed potential

**Table 1 – Overview of proteomics studies in animal models of asthma and COPD.**

| Reference | Species | Disease | Inducer | Intervention | Sample | Technique | Main findings | Up/down[a] |
|---|---|---|---|---|---|---|---|---|
| Houtman et al. [42] | Mouse | Non-allergic asthma | DNFB | | Lung | 2D-GE<br>Silver staining | Coronin 1A<br>Vinculin<br>Gelsolin | ↑<br>↑<br>↓ |
| Roh et al. [43] | Mouse | Allergic asthma | OVA | DMS | Lung | 2D-GE<br>Silver staining | T complex polypeptide 1<br>EH-domain containing protein 4<br>Plasminogen | ↑<br>↓<br>↓ |
| Signor et al. [19] | Rat | Allergic asthma | OVA | Endotoxin | BAL | 2D-GE<br>Coomassie staining | Fetuin A<br>Fetuin B<br>Haptoglobin<br>Clara cell 10 kDa secretory protein | ↑<br>↑<br>↑<br>↓ |
| Jeong et al. [45] | Mouse | Allergic asthma | OVA | | Lung | 2D-GE<br>Coomassie staining | Chitinase 3-like 3<br>Chitinase 3-like 4 | ↑<br>↑ |
| Zhao et al. [60] | Mouse | Allergic asthma | OVA | | BAL | 2D-GE<br>Silver staining | Lungkine<br>Chitinase 3-like 3<br>Chitinase 3-like 4<br>Calcium-activated chloride channel regulator 1 | ↑<br>↑<br>↑<br>↑ |
| Greenlee et al. [50] | Mouse MMP2$^{-/-}$ MMP9$^{-/-}$ | Allergic asthma | OVA | | BAL | 2D-DIGE | Chitinase 3-like 3<br>Calgranulin A | ↑<br>↑ |
| Zhao et al. [36] | Mouse | Allergic asthma | OVA | DMS | BAL | 2D-GE<br>Silver staining | Chitinase 3-like 3<br>Chitinase 3-like 4<br>Vitamin D binding protein<br>Hemopexin | ↓<br>↓<br>↑<br>↓ |
| Liu et al. [61] | Mouse | Allergic asthma | OVA | Salbutamol | Lung | 2D-GE<br>Silver staining | Rho GDP-dissociation inhibitor 2<br>Peroxiredoxin 5 | ↑<br>↑ |
| Zhang et al. [25] | Rat | COPD | Cigarette smoke | | Lung | 2D-GE<br>Coomassie staining | Thioredoxin<br>Peroxiredoxin 6<br>α-Enolase | ↑<br>↑<br>↓ |
| Calvo et al. [23] | Mouse | Allergic asthma | OVA | | Lung | SELDI-TOF MS | Found inflammatory zone 1<br>Calcyclin<br>Clara cell 10 kDa secretory protein | ↑<br>↑<br>↑ |

**Table 1 (*Continued*)**

| Reference | Species | Disease | Inducer | Intervention | Sample | Technique | Main findings | Up/down[a] | |
|---|---|---|---|---|---|---|---|---|---|
| Xu et al. [26] | Rat | COPD | Cigarette smoke Radon | | Lung | 2D-GE Coomassie staining | RAGE | ↑ | |
| | | | | | | | Thioredoxin | ↑ | |
| | | | | | | | Calcyclin | ↑ | |
| Haenen et al. [20] | Mouse | Occupational asthma | TDI | | Aur LN BAL Serum | 2D-DIGE | Lymphocyte specific protein 1 | ↓ | |
| | | | | | | | Vitamin D binding protein | ↑ | |
| | | | | | | | Hemopexin | ↑ | |
| Calvo et al. [24] | Mouse | Allergic asthma | OVA | | Lung | 2D-DIGE | 78 kDa glucose-regulated protein precursor | ↑ | |
| Tewari et al. [35] | Mouse | COPD | Cigarette smoke | | Plasma | 2D-DIGE | Fibrinogen | ↓ | |
| | | | | | | | α-1-antitrypsin | ↑ | |
| | | | | | | | Arginase | ↓ | |
| Louten et al. [21] | Mouse | TSLP Tg mice | – | | BAL | 1D-GE | Chitinase 3-like 3 | ↑ | |
| | | | | | | | Chitinase 3-like 4 | ↑ | |
| | Macaque | | HDM | | BAL | LC-MS/MS | Cyclophilin A | ↑ | |
| | | | | | | | Cofilin A | ↑ | |
| Haenen et al. [41] | Mouse | Occupational asthma | TDI | | Aur LN Serum | 2D-DIGE | | 1 sens | 2 sens |
| | | | | | | | Coronin 1a | ↑ | ↑ |
| | | | | | | | Lymphocyte specific protein 1 | – | ↓ |
| | | | | | | | Hemopexin | ↑ | ↓ |

[a] Differences presented are always comparisons from a certain disease vs. healthy (e.g. asthma vs. control).
Identification of differentially expressed proteins was performed via mass spectrometry (MALDI-TOF MS or tandem mass spectrometric analysis).
*Abbreviations*: 1D-GE, one-dimensional gel electrophoresis; 2D-GE, two-dimensional gel electrophoresis; 2D-DIGE, two-dimensional difference gel electrophoresis; Aur LN, auricular lymph nodes; BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; DMS, dexamethasone; DNFB, dinitrofluorobenzene; EH-domain, epidermal growth factor receptor substrate 15; HDM, House Dust Mite; LC–MS/MS, liquid chromatography mass spectrometry; MMP, matrix metalloproteinase; OVA, ovalbumin; RAGE, receptor for advanced glycation endpoints; SELDI-TOF MS, surface enhanced laser desorption/ionization – time of flight mass spectrometry; sens, sensitization; TDI, toluene-2,4-diisocyanate; TSLP Tg, thymic stromal lymphopoietin trangenic.

**Table 2 – Overview of proteomics studies in human asthma and COPD.**

| Reference | Disease | Sample | Technique | Main findings | Up/down[a] |
|---|---|---|---|---|---|
| Merkel et al. [28] | COPD | BAL | SELDI-TOF MS | Neutrophil defensin 1 | ↑ |
| | | | | Neutrophil defensin 2 | ↑ |
| | | | | Calgranulin A | ↑ |
| | | | | Calgranulin B | ↑ |
| | | | | Clara cell phospholipid-binding protein | ↓ |
| Candiano et al. [44] | Allergic asthma | Bronchial epithelial cells *(IL-4 stimulated)* BAL | 2D-GE Coomassie staining | Gelsolin (45 kDa) | ↑ |
| | | | | Gelsolin (85 kDa) | ↑ |
| | | | | Gelsolin (45 kDa) after allergen challenge | = |
| | | | | Gelsolin (85 kDa) after allergen challenge | ↓ |
| Steiling et al. [29] | COPD | Bronchial epithelial cells | 1D-GE LC–MS/MS | PLUNC | ↓ |
| | | | | Clara cell 10 kDa secretory protein | ↓ |
| | | | | Prolyl 4-hydroxylase beta subunit | ↑ |
| Larsen et al. [62] | Mild asthma | BAL | 2D-GE Coomassie or silver staining | Haptoglobin | ↑ |
| Larsen et al. [46] | Mild asthma Scleroderma (SSc) | Fibroblasts cultured in BAL & bronchial biopsies | 2D-GE Coomassie or silver staining | Alpha-smooth muscle actin | ↑ |
| Jeong et al. [47] | Allergic asthma | Peripheral T lymphocytes | 2D-GE Silver staining | Phosphodiesterase 4 | ↑ |
| | | | | Thioredoxin 2 | ↑ |
| | | | | Glutathione S transferase M3 | ↓ |
| Ohlmeier et al. [48] | COPD | BAL | 2D-GE Silver staining | Surfactant protein A | ↑ |
| Gray et al. [27] | Allergic asthma COPD CF | Sputum | SELDI-TOF MS | Calgranulin A, B & C | ↑ |
| | | | | Clara cell 10 kDa secretory protein | ↓ |
| | | | | Proline rich salivary peptide | ↓ |
| Wu et al. [22] | Allergic asthma | BAL after segmental airway challenge | LC–MS/MS | Matrix metalloproteinase-9 | ↑ |
| | | | | Calgranulin B | ↑ |
| | | | | Tissue inhibitor of metalloproteinases 1 | ↑ |
| Bandow et al. [63] | COPD | Plasma | 2D-GE Sypro Ruby | Plasma retinal-binding protein | ↓ |
| | | | | Glutathione peroxidase 1 | ↑ |
| | | | | Fibrinogen | ↑ |
| | | | | Apolipoprotein E | ↑ |
| Bozinovski et al. [64] | COPD | Serum | SELDI-TOF MS | Serum amyloid A | ↑ |
| Hur et al. [37] | Occupational asthma | BAL | 2D-GE Coomassie staining | Ferritin | ↓ |
| | | | | Transferrin | ↑ |
| Lee et al. [33] | COPD | Lung tissue | 2D-GE Silver staining | Matrix metalloproteinase 13 | ↑ |
| | | | | Thioredoxin-like 2 | ↑ |
| Gomes-Alves et al. [65] | Allergic asthma COPD CF | Serum Nasal epithelial cells | SELDI-TOF MS | Biomarker signatures, one significant identification, hemoglobin subunit beta (↑ in CF as compared to asthma & control) | |
| Bloemen et al. [58] | Allergic asthma | EBC | LC–MS/MS | Proteolytic peptide profiles discriminate between healthy vs. asthma. No identifications so far | |

**Table 2 (*Continued*)**

| Reference | Disease | Sample | Technique | Main findings | Up/down[a] |
|---|---|---|---|---|---|
| Gharib et al. [59] | Allergic asthma | Sputum | LC–MS/MS | Calgranulin A | ↓ |
| | | | | Calgranulin B | ↓ |
| Hu et al. [32] | COPD | Lung tissue | 2D-GE | Heat shock protein 27 | ↑ |
| | | | MALDI-TOF MS | Cyclophilin A | ↑ |
| Ohlmeier et al. [66] | COPD | Sputum | 2D-DIGE | Polymeric immunoglobulin receptor | ↑ |
| Verrills et al. [34] | COPD | Plasma | 2D-DIGE | α2-macroglobulin | ↓ |
| | Allergic asthma | | | haptoglobin | ↑ |
| | | | | ceruloplasmin | ↓ |
| | | | | hemopexin | ↓ |
| Alexandre et al. [56] | COPD | Erythrocyte | $O^{16}/O^{18}$ labeling | Chorein | ↓ |
| | | membrane proteins | LC–MS/MS | Methemoglobin reductase | ↓ |
| Franciosi et al. [31] | COPD | Epithelial lining fluid | iTRAQ | Lactotransferrin | ↑ |
| | | | 2D-LC–MS/MS | Cofilin-1 | ↑ |
| | | | MALDI-TOF/TOF MS | HMGB1 | ↓ |
| | | | | Alpha 1-antichymotrypsin | ↑ |
| Lee et al. [67] | Uncontrolled asthma | Sputum | 2D-GE | S100 calcium binding protein A9 | ↑ |
| | | | Coomassie staining | | |
| Merali et al. [68] | COPD | Plasma | 1D-GE | Glucose regulated protein 78 | ↑ |
| | | | LC–MS/MS | Soluble hemoglobin scavenger receptor | ↓ |
| | | | | IL-1 receptoraccessory protein | ↑ |
| | | | | Macrophage stimulatory protein | ↑ |
| Pastor et al. [30] | COPD | BAL | 2D-GE | Peroxiredoxin 1 | ↑ |
| | | | Sypro Ruby | Heat shock protein 70 | ↑ |
| | | | | Pyruvate kinase 2 | ↑ |
| Tu et al. [69] | COPD | BAL | LC–MS/MS | Alcohol dehydrogenase 1B | ↑ |
| | | | | Aldehyde dehydrogenase family 3A1 | ↑ |
| | | | | Aldehyde dehydrogenase (mitochondrial) | ↑ |

[a] Differences presented are always comparisons from a certain disease vs. healthy (e.g. asthma vs. control).
Identification of differentially expressed proteins was performed via mass spectrometry (MALDI-TOF MS or tandem mass spectrometric analysis).
*Abbreviations*: 2D-GE, two-dimensional gel electrophoresis; 2D-DIGE, two-dimensional difference gel electrophoresis; BAL, bronchoalveolar lavage; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; EBC, exhaled breath condensate; LC–MS/MS, liquid chromatography mass spectrometry; PLUNC, palate, lung and nasal epithelium carcinoma associated protein precursor; SELDI-TOF MS, surface enhanced laser desorption/ionization – time of flight mass spectrometry; SSc, systemic sclerosis.

protein biomarkers in blood derived samples (plasma, serum or erythrocytes). These human proteomic studies revealed significant changes in proteins involved in the regulation of inflammation [27–29], oxidative stress [30–32], the acute phase and immune response [31–35] and structural proteins [31,33]. The highlighted proteins in all these studies could be defined as markers of established disease. They are, however, less suited for the detection of early disease onset [16].

The influence of steroid treatment (dexamethasone) on the proteome profile of BAL fluid obtained from murine models, has also been investigated. The level of several proteins, known to be upregulated in allergic asthma, such as chitinase 3-like 3 (Ym1), chitinase 3-like 4 (Ym2) and surfactant protein D were decreased after dexamethasone treatment [36]. Louten et al. [21], using a transgenic mouse model which spontaneously develops asthma, confirmed some of the results. In this study, Ym1 and Ym2 were upregulated both at the protein and mRNA level, comparing control versus asthmatic mice. After dexamethasone treatment, *Ym1* mRNA levels decreased whereas *Ym2* mRNA levels were not affected by the treatment.

Although most studies have been performed in established allergic asthma (or COPD), a reasonable subset of asthma (9–15%) is attributable to occupational exposures (e.g. animal derived proteins, flour, chemicals, etc.). Only few studies have investigated proteomic changes in occupational asthma. One study by Hur et al. [37] detected significant differences in ferritin and transferrin levels in BAL fluid and serum of diphenylmethane diisocyanate (MDI) asthmatic workers compared to MDI-exposed asymptomatic workers and non-atopic healthy controls. Unfortunately, these results were not confirmed in a more recent study by Sastre et al. [38], investigating workers exposed to toluene diisocyanate (TDI). In one of our studies, we found changes in the levels of ferritin and transferrin in BAL fluid of TDI-asthmatic mice, as shown by Hur et al. The discrepancy between the results obtained by Hur et al. and Sastre et al. concerning biomarkers of diisocyanate asthma are currently a matter of debate since clinical cross-reactivity between diisocyanates (TDI and MDI) is suggested to be common [39]. Moreover, both the study of Hur et al. and Sastre et al. used populations with similar characteristics, although Hur et al. had a considerably larger group of asymptomatic and healthy controls compared to Sastre et al.

Asthma phenotypes share commonalities in their pathology such as oxidative stress, inflammation and non-specific airway hyperreactivity. Nevertheless, some of the proteins such as peroxiredoxins and enolases, are commonly identified regardless of the studied pathology, implying that caution should be taken when attributing the term 'biomarker' to a certain protein [40].

So far, proteomic studies conducted in pulmonology have investigated changes in the proteome of healthy subjects versus diseased individuals (asthma, COPD, etc.) using samples from either animal models or patients. Results from a study of our own group in a mouse model of chemical-induced asthma, show that already after two sensitizations, substantial differences occur in the proteome, which we presume relate to the onset of an immune response leading to asthma [41].

## 3. Methodology

Most often 2D-gel electrophoresis (2D-GE) combined with silver or Coomassie staining and a software package, has been used to separate, visualize and analyze differences in protein levels between healthy and diseased subjects (Tables 1 and 2) [26,36,37,42–48]. This is followed by peptide mass fingerprinting or tandem mass spectrometry analysis for the identification of the proteins. The major drawbacks of silver and Coomassie staining are the small linear dynamic range along with the high need for many technical and biological replicates – to correct for the variation between different stained gels – as well as the limited sensitivity of these methods.

The introduction of two-dimensional difference gel electrophoresis (2D-DIGE) has overcome these shortcomings and is now the gold standard among gel-based differential proteomics techniques [49]. This fluorescence-based technique allows the use of multiplexed samples with an internal standard that virtually eliminates gel-to-gel variability, increasing the confidence that uncovered differences are due to real changes, rather than inherent biological variation or experimental variability. Some recent studies in non-malignant lung diseases have applied 2D-DIGE rather than the less preferred silver staining method [20,24,50]. Although 2D-DIGE revolutionized differential analysis in proteomics, this gel-based technique also has some inherent limitations. Particularly, proteins present in extremely low concentrations or proteins that cannot be separated via gel-based techniques due to their physicochemical properties (iso-electric point, hydrophobicity, molecular weight) will not be detected [51,52]. Membrane bound proteins such as surface markers, which could be important in the development of asthma and COPD, are not resolved via gel-based proteomics techniques due to their hydrophobic nature.

Several gel-free proteomics technologies were developed to overcome these limitations. Surface enhanced laser desorption/ionization mass spectrometry (SELDI-TOF) is suited to directly profile subsets of smaller proteins (<20 kDa) based on their physicochemical properties (hydrophobic, metal-binding, etc.) [23,27], however the poor resolution often hampers identification of the proteins.

In other gel-free methods, a protein mixture is enzymatically cleaved into peptides, and subsequently these peptides are separated and identified using liquid chromatography and tandem mass spectrometry (LC–MS/MS) [58,59]. Relative quantitation can be derived by direct comparison of ionic signals produced by peptides in parallel LC–MS analyses. This label-free method uses statistical tools to identify peptides that express consistent differences between samples across multiple runs.

An alternative for relative quantitative analysis is tagging the peptides with chemically almost identical labels that have a mass difference (isotopes). The samples are then combined and analyzed together using LC–MS. The relative abundance of the peptide in the different conditions can be calculated from the ratio of the peak intensity of the different isotopic forms [53]. Isobaric tags for relative and absolute quantification (iTRAQ) is based on covalent labeling of the

N-terminus and side chain amines of peptides from protein digestions with tags of varying mass. Combined with high resolution LC–MS/MS iTRAQ allows the quantitative comparison of protein levels. Franciosi et al. [31] successfully applied this technique in epithelial lining fluid of COPD patients and non-COPD controls and found significantly different levels of lactotransferrin, high-mobility group protein B1, alpha 1-antichymotrypsin and cofilin-1. Stable isotopic labeling by amino acids in cell culture (SILAC) involves the metabolic incorporation of 'normal' or 'heavy' forms ($C^{13}$ or $N^{15}$) of arginine or lysine, supplied in culture medium, in newly synthetized proteins. The normal and heavy forms of the peptides appear as two distinct peaks in MS and relative quantitation is achieved by comparing the signal intensities. SILAC has been successfully used in studies focusing on lung cancer [54,55]. Alexandre et al. [56], used differential oxygen ($O^{16}/O^{18}$) labeling to quantify differences in erythrocyte membrane proteins of COPD patients compared to healthy controls. Differential $O^{16}/O^{18}$ labeling occurs at the C-terminal carboxyl group of peptides, derived from the proteolytic digestion of proteins, where two $O^{16}$ atoms are replaced by $O^{18}$ atoms. The resulting mass shift permits the relative quantitation of proteins between two conditions [57].

These gel-free methods also have their inherent limitations. A typical tissue extract contains a few thousands of proteins that after digestion will result in a more complex peptides mixture, while the number of peptides that can be identified due to co-elution and ion suppression is limited [58]. Posttranslational modifications such as phosphorylations, often important in the regulation of protein function, will be visualized on 2D gels by different spots as a result of shift in isoelectric point (pI). In gel-free proteomics, pI information, together with protein molecular weight information, is lost.

## 4. Mice vs. men

One can debate if human samples are preferable over samples obtained from mouse models. Nonetheless, significant understanding on the pathogenesis of asthma and COPD has been derived from animal experiments, particularly in mice and rats [25,26,70]. Several mouse specific immunological tools and the availability to use transgenic mice (e.g. TSLSP Tg mice) favor this species [21]. Moreover, 99% of mouse genes have homologues in humans, facilitating proteomics approaches [71]. Also, ethical and moral issues prevent profound mechanistic investigations in humans [72]. Animals are generally kept in clean animal houses, limiting the environmental factors to which they are exposed and facilitate the exposure to exact concentrations of a known substance (e.g. allergen or tobacco smoke) [73]. In animals, invasive samples, such as lung tissue, lymphoid tissue and BAL fluid are more easily obtained than in humans.

Unfortunately, the results obtained from animal experiments cannot directly be extrapolated to humans since important differences in airway morphology and development exist between mice and humans. Moreover, the initiation, development and responses of the innate and adaptive immune system differ substantially between mice and men [74]. No mouse model is currently available that matches all phenotypic characteristics of human asthma [75–77], nor COPD [78]. Most mouse models are an acute model of asthma, lacking chronicity and airway remodeling, two characteristics of human asthma. The choice of mouse strain has also been demonstrated to have an influence on the physiological parameters (cytokines, neutrophils, eosinophils, etc.) [79–81]. Yet, it is possible to induce several characteristics of the disease such as lymphocyte activation and IgE production, along with airway hyperresponsiveness, airway inflammation and in some studies airway remodeling [24,82–87].

Bearing in mind these shortcomings, animal models can be seen as a platform to generate and test hypotheses which need validation in humans [88]. Despite the significant results obtained from animal studies, translation to human disease is difficult and time consuming. Hackam et al. [11] have reviewed highly cited animal studies, showing that only one out of three animal studies evaluating medical interventions for human diseases have been replicated in clinical trials.

## 5. Sample origin

Another important issue concerns the best choice of tissue for analysis. So far, lung tissue has been the most used sample in murine models of asthma and COPD for obvious reasons [23,24,26,42,43,45,89]. In animals, it is possible to obtain samples requiring more invasive techniques and, moreover, lung tissue will possibly contain high concentrations of potential biomarkers specific for lung diseases. Nevertheless, studying proteome changes in lung tissue by gel-based techniques is limited to the soluble proteins. It is important to validate the biomarkers obtained in lung tissue, using more accessible samples, such as BAL fluid and to see how well data of both compartments are correlated [90]. BAL fluid provides the most faithful reflection of the protein composition of the pulmonary lumen and is therefore the preferred sample in humans [91–93]. However, proteomic analysis of BAL fluid is complicated by a low protein abundance and high salt content and possible contamination by serum proteins, as a result from leakage during lung inflammation [94]. Although serum proteins in BAL could also reflect the current pulmonary situation, albumin and immunoglobulin depletion strategies have been developed and applied to improve the quality of proteomic studies [20].

Recently, in humans, the use of less invasive techniques such as induced sputum and exhaled breath condensate (EBC) have also been evaluated [27,58]. Induced sputum and EBC can be routinely collected from patients during follow-up, but the use of these samples in proteomics studies is scarce due to the very low protein content and needs to be optimized.

Strikingly, only few of the studies listed in Tables 1 and 2 used plasma or serum samples [20,41,63,64]. Serum is an ideal biological sample that contains an archive of information due to the presence of a variety of proteins released by diseased tissue [95]. Nevertheless, proteomic analysis of serum has several advantages and disadvantages. Serum is easy to acquire, can be obtained at different time points (e.g. during follow-up) and has a high protein content. However, high-abundant proteins (albumin, IgG and transferrin) can possibly mask potential

biomarkers with a relative low abundance, and serum is also highly variable [96].

It seems that the samples with the highest protein content show fewer significant differential proteins, giving the impression that the complexity reduces the sensitivity. Therefore, enrichments of subsets of cells, such as T- and B-lymphocytes, or macrophages and leucocytes, could reduce the complexity and facilitate proteomic analysis. On the other hand, a lot of starting material (*cfr*. mice) is required to have enough cells to perform these experiments, which is a major limiting factor. In humans, this can be overcome because a large amount of blood can be sampled to isolate specific lymphocyte subsets, as done by Jeong et al. [47], who compared human peripheral T-lymphocytes from asthma patients and healthy controls.

## 6. Interpretation of results

Most often, the end-result of a proteomic study is the identification of ten to hundreds of differentially expressed proteins which are presented in a table. A few of these proteins are selected based on literature and biological relevance. Several proteins, such as annexins, peroxiredoxins and haptoglobins, are differentially expressed in different studies when comparing all the results of the papers described in Tables 1 and 2. One should question if these proteins could be called biomarkers since these proteins are repeatedly identified throughout different research domains and are therefore called 'déjà-vu'-proteins [40]. Chitinase 3-like 3 (Ym1) and Chitinase 3-like 4 (Ym2) are both members of the chitinase family, responsible for the degradation of chitin which is present in the exoskeleton of arthropods and in cell membranes of fungi. Although chitin is not present in mammalians they are regularly associated with asthma in murine models [21,45,60]. Although no human orthologues have been identified for Ym1 and Ym2, other human chitinases have been demonstrated to be involved in the pathogenesis of allergic disease and COPD.

In summary, drawing general conclusions from common identified proteins is challenging, because of the variety of mouse strains, tissues and treatment protocols (difference in timing, dosage and duration) that are used to induce asthma or COPD (e.g. acute versus chronic). In addition, substantial differences in physiology between mice and man set hurdles in extrapolating the results obtained from murine models to humans.

## 7. Future

The rationale for choosing proteomics approaches, is to discover novel target proteins as biomarkers for disease identification, as well as possible new markers with the focus on refining treatment. Although different proteins are highlighted in Tables 1 and 2 that could be linked with the phenotypical characteristics of asthma or COPD in murine models and humans, functional biological validation – specifically for murine studies - is nearly always lacking, thereby indicating the long way that is still to go in proteomics biomarker research. One exception is the 2D-DIGE study of lung tissue of Calvo et al. [24], who discovered the involvement of a 78 kDa glucose-regulated protein precursor (Grp78) in OVA-induced asthma in mice. They silenced down the gene responsible for this protein using anti-Grp78 siRNA treatment in OVA-sensitized and challenged mice, resulting in decreased airway hyperreactivity (AHR) and eosinophilic inflammation. This type of validation is valuable in determining the biological relevance of the studied proteins as possible therapeutic targets. On the other hand, proteomics studies are often used to screen for markers of disease. Clinically useful biomarkers would provide earlier and better diagnosis of patients, permitting treatments to be initiated in early stages of the disease, when the chance of success is greatest [96,97]. Several proteins such as ferritin, transferrin and calcyclin were proposed as general markers of lung disease [23,37]. As already indicated, Hur et al. described ferritin and transferrin as possible biomarkers of MDI-induced asthma, but Sastre et al. could not verify these markers. Moreover, ferritin and transferrin are involved in a broad range of biological processes, limiting their use as specific biomarkers. More efforts should be focused to find disease-specific, unique biomarkers for lung diseases. Perhaps future efforts should be less dedicated to known proteins found differentially in these studies, but more to explore the proteins with unknown ontology. This could lead to really novel and innovating biomarkers and perhaps also therapeutic targets.

Furthermore, current state-of-the-art diagnosis and management schemes of asthma are suboptimal since the incidence of asthma has risen by 250% over the last two decades [16]. There is an urgent need for early diagnosis since asthma generally starts early during childhood. Occupational asthma often is exclusively related to exposures on the work floor and thereby only develops at a later stage of life. However, the onset of allergic OA is always preceded by a complaints-free latency period in which workers are getting sensitized, as a result of complex immunological and molecular processes that are not identified by currently used diagnostic tools but which could be picked up by '-omics' techniques.

In addition, peptide hormones, neuropeptides, cytokines and chemokines are involved in the regulation of numerous physiological processes and the onset, maintenance and progression of diseases such as asthma. These peptides are generally not detected by proteomics techniques either due to their size (<20 kDa) or to their low abundance and therefore are a yet unexplored pool of possible new biomarkers [98]. Peptidomics techniques, using a combination of liquid chromatography and mass spectrometry can be used to identify peptides. Nonetheless, quantitative peptidomics is still a major challenge.

## 8. Conclusion

There is an increasing trend in the use of proteomics to explore new biomarkers of asthma or COPD. Although different murine models have been developed to study these lung diseases, and proteomics studies have been performed, functional validation of the identified candidate biomarkers or translation into clinical studies is often lacking. Shifting the focus from disease toward the early events during disease manifestation (e.g. sensitization) and the investigation of peptides as a yet unexplored pool of interesting biomarkers

would bear great potential in the identification of possible new, clinically useful biomarkers.

## Conflict of interest statement

The authors declare that they have no conflicts of interest.

## Transparency document

The Transparency document associated with this article can be found in the online version.

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